Accumulation of β-catenin protein, mutation in exon-3 of β-catenin gene and loss of heterozygosity in solid pseudopapillary tumor of the pancreas

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Directed by Professor Seung Hoon Choi

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감사의 글

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우선 본 논문시작부터 완성까지 지도와 격려를 아끼지 않았던 지도교수 최승훈 교수님께 진심으로 감사드립니다. 아울러 이우정, 김호근, 송시영, 라선영 교수님께도 감사의 말을 전합니다.

한 실험실에서 공부하고 힘들 때나 기쁠 때 늘 같이 하던 박미경, 조은영, 박기청에게 감사드립니다.

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2004 년 12 월

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Accumulation of β-Catenin Protein, Mutation in Exon-3 of β-catenin Gene and Loss of Heterozygosity in Solid Pseudopapillary Tumor of the Pancreas

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Solid pseudopapillary tumors (SPTs) are rare pancreatic neoplasms of low malignant potential, and the molecular events contributing to their genesis remain unknown. To better understand the factors leading to human SPT development, we examined β-catenin mutation, nuclear accumulation, and loss of heterozygosity (LOH) in coexisting normal pancreatic tissues and SPTs. We microdissected 20 cases of SPTs and 18 cases of coexisting histologically normal pancreatic tissues, and examined a mutation in exon 3 of β-catenin and LOH at 10 microsatellite markers on 9 chromosome arms

using genomic DNA. At the same time, immunohistochemistry was performed for β-catenin. Activating mutations in codons 32 to 37 of β-catenin exon-3 were present in 16 of 20 (80%) SPTs. Allelic loss on chromosome 5q 22.1 was present in 10 of 18 (55.5%) cases, while no allelic losses were found on chromosomes 1p, 6q, 9p, 9q, 11p, 11q, 17p, or 22q. Nuclear accumulation of β-catenin was demonstrated in all 20 cases (100%). In contrast, there were no β-catenin exon-3 mutations, no nuclear accumulations, or no allelic losses in the corresponding normal tissues. The presence of a β-catenin exon-3 mutation and nuclear accumulation in SPTs suggest that these mutations and accumulations arise in tumorigenesis. Finally, frequency of allelic loss on chromosome 5q 22.1 suggests that a locus on DS592 is involved in the molecular pathogenesis of SPTs.

Key words: Solid pseudopapillary tumor of pancreas, β-catenin ,mutation, immunohistochemistry, loss of heterozygosity.

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I. INTRODUCTION

Solid pseudopapillary tumors (SPTs) of the pancreas are uncommon tumors, comprising only ~1% of pancreatic neoplasms.[1] SPTs are histologically, clinically, and prognostically quite distinct from other pancreatic neoplasms.[2] Unlike the infiltrative growth and almost invariably lethal behavior of ductal adenocarcinmas among older individuals, SPTs are neoplasms of young women and are frequently presented as circumscribed

cystic lesions.[3] Histologically, the uniform tumor cells showed diffuse and strong positivity for vimentin, Neuron-specific enolase (NSE), and the progesterone receptor, and occasionally faint staining for synaptophysin cytokeratins (CKs) 8 and 18. In addition, they show nuclear positivity for β-catenin. They are negative for Carcinoembryonic Antigen (CEA) and trypsin. About 10% are malignant, metastasizing to the peritoneum and/or the liver.[4, 5]

Loss of heterozygosity (LOH) is one of the most important mechanisms for inactivation of tumor suppressor genes and is involved in almost all carcinogenesis.[6-9] Currently, a broad spectrum of distinct gene mutations and chromosomal alterations in pancreatic neoplasms have been defined.[10-12] The known genetic anomalies in ductal, intraductal, and papilla of Vater neoplasia have been described previously. However, owing to low incidence, the data are limited regarding molecular abnormalities of acinar cell, serous cystic, mucinous cystic, and solid pseudopapillary tumors. In recent years, the significance of alterations in the APC/β-catenin pathway in the tumorigenesis of SPTs has been studied, and the mutations at exon 3 of β-catenin have been found in some patients. However, alterations of chromosomes are unknown, and allele loss has not been defined.[13, 14]

II. MATERIALS AND METHODS

Case and Tissue Selections

Table 1 shows the demographics of the 20 patients (17 females, 3 males; mean, 26.7 years; range, 11-59 years) with SPTs whose tumors were analyzed in this study. All tumors were completely resected and had no metastases. Tumor samples were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. Sections of 4 μm thick were stained with H&E. Histopathological diagnosis was confirmed by pathologists.

Immunohistochemical Analysis of β-Catenin

Each tumor section was deparaffinized and subjected to antigen retrieval by microwaving in 0.1 M of citrate buffer (sodium citrate, pH 6.0) for 15-30 minutes. Sections were incubated with anti-β-catenin (Transduction Laboratories, Lexington, Ky, USA) monoclonal antibodies at a dilution of 1:500, overnight, at 4°C, and stained by the avidin-biotin complex method using a Histofine Kit (Nichirei, Tokyo, Japan). The β-catenin immunostaining results for both cytoplasm and nucleus were evaluated by comparing the staining intensities of tumor cells and adjacent nontumor epithelial cells. Sections incubated with PBS instead of the primary antibodies served as negative controls. The sections were counterstained by

Mayer's hematoxylin.

According to the criteria used by Susan C and colleagues: immunostaining labeling was evaluated for the presence of nuclear, cytoplasmic, and membranous β-catenin accumulation in the SPTs and surrounding nonneoplastic pancreas, if present. Nuclear and cytoplasmic accumulation of β-catenin in SPTs was graded according to the percentage of neoplastic cells with strong immunolabeling. Sections incubated with PBS instead of the primary antibodies served as negative controls. The sections were counterstained by Mayer's hematoxylin.

Genomic DNA Preparation

Sufficient numbers of formalin-fixed, paraffin-embedded sections were available in 20 cases. Tumor areas were microdissected from four to eight 4-μm sections, which were deparaffinized in xylene, rehydrated in ethanol, and air dried. Adjacent nontumorous tissue was found in 18 cases and was separately dissected. DNA extraction was performed by the SDS-proteinase K and phenol-chloroform extraction method. The DNA was dissolved in 20 μL of 10 mM Tris-HCL (pH 8.0).

Mutational Analysis

Genomic DNA from each SPT and normal tissue sample was

amplified by polymerase chain reaction (PCR) using the primer pair: forward, 5'-ATGGAACCAGACAGAAAAGC-3'; reverse, 5'-GCTACTTGTTCTTGAGTGAAG-3'. These amplified a 200-bp fragment of exon 3 of the β-catenin gene encompassing the region for GSK-3 phosphorylation. The PCR reactions were performed in 20 μL of reaction mixture containing 2 μL of template DNA, 2 μL of 10 x buffer, 4 μL of 5 x Q-solution, 1 μL of both 5' and 3' oligonucleotides (final concentration of 0.5 μM), 1.2 μL of 2.5 mM deoxynucleotide triphosphate mix, and 0.2 units of Tag DNA polymerase (QIAGEN Inc, Valencia, Calif, USA). The reaction mixture was preincubated for 3 minutes at 94°C and incubated for 35 cycles (94°C for 1 minute, 53°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes). PCR products were purified using QIAquick PCR purification kit (QIAGEN) before sequencing. Automated sequencing of purified PCR products was performed on an ABI prism 3100 DNA analyzer (Applied Biosystems, Foster City, Calif, USA) using the internal primers: 5'-AAAGCGGCTGTTAGTCACTGG-3'; reverse, 5'-CCTGTTCCCACTCATACAGG-3'. The resulting sequence data were analyzed with the Sequencer analysis program (Gene Codes, Ann Arbor, Mich, USA). All mutations were verified in both sense and anti-sense directions on independent PCR products.

Microsatellite Selection

Ten microsatellite markers, located on 9 chromosomal arms, were selected for utility in SPTs tissue based on the following criteria: Location at regions relevant to pancreas tumorigenesis (ie, regions of LOH in early-stage carcinomas, or at sites of identified or putative tumor suppressor genes). Markers at regions not believed relevant to pancreas tumorigenesis were also included highly polymorphic (ideally > 75% heterozygosity) and ability to be multiplexed together without adverse interaction. Chromosomal regions and markers used were as follows: 1p:D1S197; 5q:D5S592; 6q:D6S287; 9p:D9S285; 9q:D9S319; 11p:D11S1984; 11q:D11S898; 17p:D17S1828; and 22q:D22S1167. Primers were synthesized commercially by Geno Tech. Corp, (Seoul, Korea).

LOH Evaluation

Genomic DNA from each SPT and normal tissue sample was amplified by PCR at 10 microsatellite loci to evaluate the LOH. These markers included D5S592, D17S1828, D17S1832, D11S1984, D11S898, D6S287, D1S197, D9S285, D9S319, and D22S1167 (Table 2). PCR reactions were carried out in a 20-μL mixture of 1.5 mM $MgCl_2$; 20 pmol of primer; 0.2 mM each of dATP, dGTP, and dTTP; 5 μM dCTP; 1 μCi [α-^{32}P]-dCTP (3000 Ci/mmol; DuPont New England Nuclear, Boston, Mass,

USA); 50 ng sample DNA; 1 x PCR buffer; and 1.25 units of Tag polymerase (Life Technologies, Inc, Gaithersburg, Md, USA). After denaturation at 95°C for 5 minutes, DNA amplification was performed for 25-30 cycles consisting of denaturation at 95°C for 30 seconds, primer annealing at 55-60°C for 30 seconds, and elongation at 72°C for 15 seconds. PCR products were separated in 6% polyacrylamide gels containing 5.6 M urea, followed by autoradiography. LOH was considered present when there was disappearance, or at least a 50% reduction in the intensity, of a heterozygous band as compared with nonneoplastic control tissues in at least 1 informative marker.

Table 1. Clinical parameters and mutations in the β-catenin gene in the SPTs

Cases	Age/Sex	Location	Nuclear β-catenin (%)	5qLOH	β-catenin gene mutation		
					Mutated Codon	Base change	Amino acid substitution
1	20/M	Body,tail	>90	+	33	TCT→CCT	Ser→Pro
2	13/F	Body,tail	>80	-		-	-
3	21/M	Body	>90	+	33	TCT→TGT	Ser→Cys
4	40/F	Head	>90	-	37	TCT→TTT	Ser→Phe
5	19/F	Body	>80	-	33	TCT→TGT	Ser→Cys
6	43/F	Head	>90	+	32	GAC→GGC	Asp→Gly
7	28/F	Head	>70	N/D		-	-
8	39/M	Tail	>80	-	33	TCT→CCT	Ser→Pro
9	32/F	Tail	>90	+	32	GAC→GGC	Asp→Gly
10	28/F	Head	>90	+	37	TCT→TGT	Ser→Cys
11	17/F	Body	>90	+	32	GAC→GGC	Asp→Gly
12	19/F	Tail	>70	-	33	TCT→CCT	Ser→Pro
13	26/F	Head	>90	+	33	TCT→TTT	Ser→Phe
14	38/F	Tail	>90	+	37	TCT→TGT	Ser→Cys
15	23/F	Head	>50	-		-	-
16	34/F	Tail	>80	-	32	GAC→GGC	Asp→Gly
17	59/F	Body	>90	+	37	TCT→CCT	Ser→Pro
18	11/M	Body	>90	N/D	33	TCT→TGT	Ser→Cys
19	19/F	Tail	>30	-		-	-
20	25/F	Tail	>90	+	32	GAC→GGC	Asp→Gly

Note: Locations of mutations in exon-3 of β-catenin are shown by codon. Nuclear accumulation was evaluated based on the percentage of strongly staining tumor cell nulcei.

Abbreviations: N/D, not performed due to lack of normal tissue for LOH;

Serine, Ser; Proline, Pro; Cysteine, Cys; Phenylalanine, Phe; Aspartic acid, Asp; Glycine, Gly;

Table 2 . Primers for LOH analysis

	Forward primer	Reverse primer	Product size
D5S592	AGACAGACAGAGAGATTAGA	AGTAAAGTGAGTGGAGAGC	145-175 (bp)
D17S1828	TGCACTCACAGATTTGCC	TTAAGCCAGTTCGGATTTG	207-227 (bp)
D17S1832	ACGCCTTGACATAGTTGC	TGTGTGACTGTTCAGCCTC	151-195 (bp)
D11S1984	GGGTGACAGAGCAAAATTCT	ACACCTGGATCTTGGACTCA	170-202 (bp)
D11S898	AGCACCATTTGCTGAGACTG	TGTATTTGTATCGATTAACCAACTT	140-156 (bp)
D6S287	ATATTAGTGCCTTATGCTTCTG	AAATTGGATATTCATGCTTG	143-171 (bp)
D1S197	TCATGTCCCTCCTCCCAAAG	GAGCAAGCATCCAAAAACGA	115-129 (bp)
D9S285	TGCCAANAGAGTAGATCTGAAG	ACCGCAATCAAGCCAAT	107-129 (bp)
D9S319	GCCAGTGTTCTCCAGAGAAA	TGGGATATGTCAGCCAAAAT	173 -190(bp)
D22S1167	ACATGGCAAAACCCAGTCTC	GGGGCTTCAACAACATTCTTAAC	266-278 (bp)

III. RESULTS

A summary of the clinicopathologic and molecular findings in the 20 SPTs ,designated 1 to 20 is presented in Table 1.

Clinicopathologic Characteristics and Conventional Histopathology

Location of the tumors was head (30%), body (35%), and tail (35%). The histologic diagnosis of SPTs was evaluated by surgical resection in all the cases. All tumors were well-encapsulated without adhesions or metastases to other organs. H&E stains of each tumor sample showed typical histopathology of SPT, characterized by growth of uniform polygonal cells arranged with minute fibrovascular stalks, exhibiting a pseudopapillary pattern (Figure 1). The tumors also showed a solid monomorphous pattern, sclerosis, and cystic degeneration of varying degrees.

Immunohistochemistry

Intense β-catenin immunoreactivity was found in the cytoplasm and nuclei of almost all tumor cells examined (Table 1). Membranous reactivity was inconspicuous. Meanwhile, acinic cells, duct cells, and some islet cells in the nonneoplastic pancreatic parenchyma showed only membranous reactivity (Figure 2).

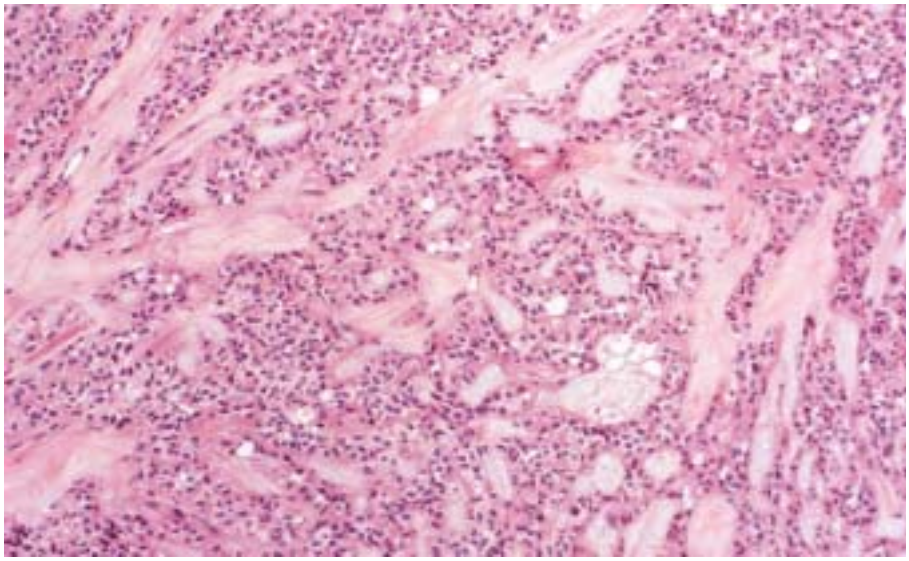
Mutational Analysis of β-catenin

Sixteen of 20 tumors (80%) showed 1-bp missense mutations in the region between codon 32 and 37 of exon 3 of the β-catenin gene. The involved codons were as follows: codon 32, 33, and 37. No mutations were found in any corresponding nontumorous tissue (Figure 3).

LOH

Allelic loss assays on chromosome 5q 22.1 revealed LOH in 10 of 18 (55.5%) patients. However, no allelic loss was found in any of the other microsatellite loci (Figure 4).

a.



b.

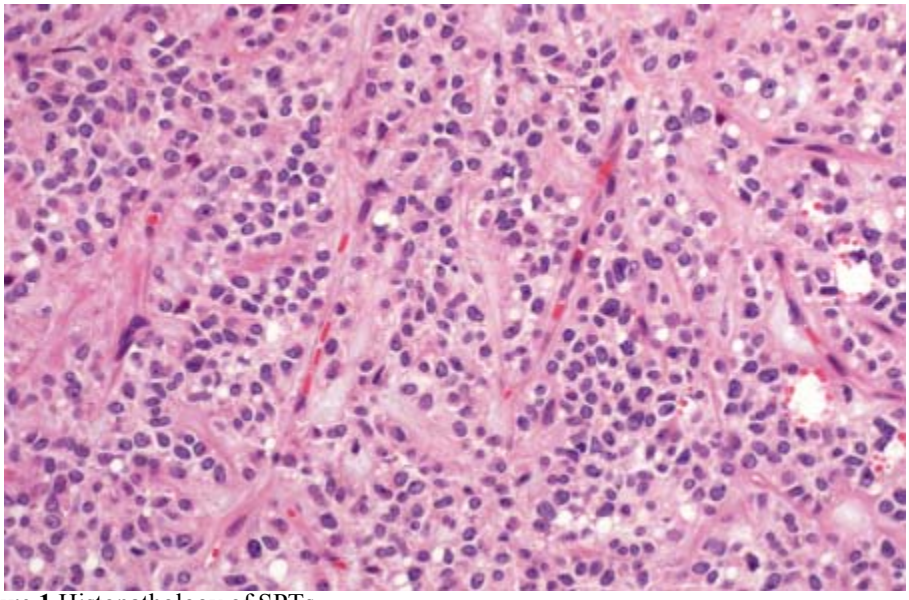
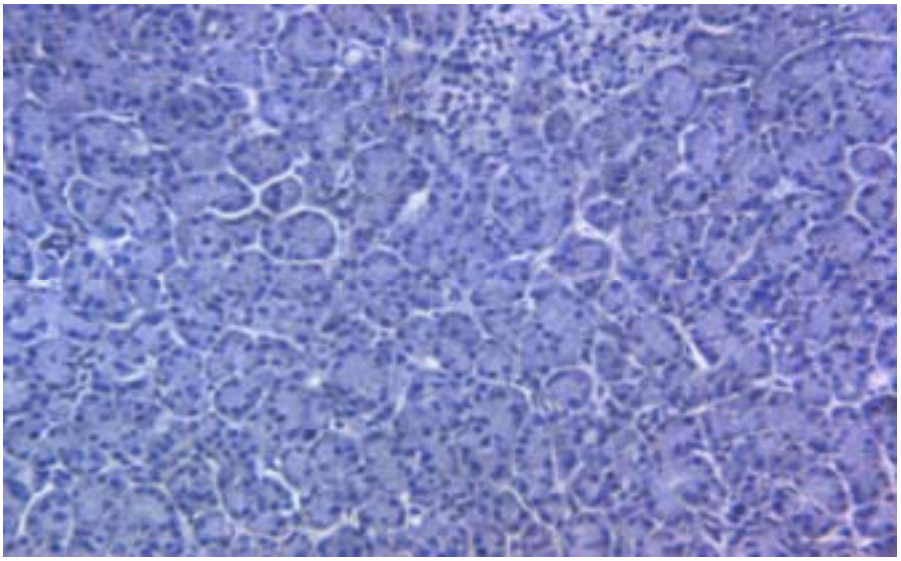


Figure 1 Histopathology of SPTs

a: Pseudopapillary pattern with one to two layers of tumor cells lining elongated fibrovascular stalks.(H &E stain, 40X)

b. Solid pattern comprising sheets of polyhedral tumor cells with regular round to oval nuclei with dispersed chromatin and small nucleoli. (H &E stain, 100X)

a.



b.

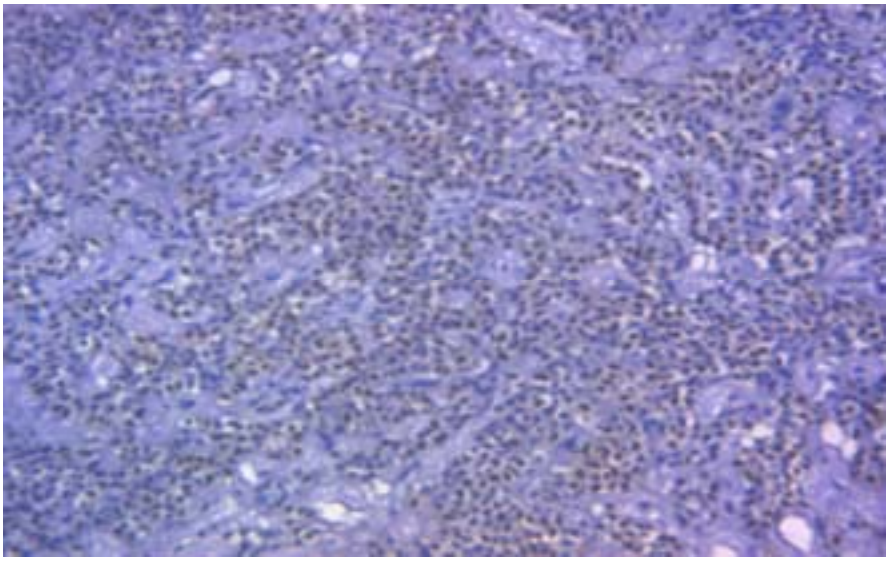


Figure 2 . Immunostainning with β-catenin

a. β-catenin immunostain in normal pancreatic tissue. Acinal cells, duct cells, and some islet cells in the nonneoplastic pancreatic parenchyma showed only membranous reactivity.

b. β-catenin immunostain in solid pattern areas. Note cytoplasmic and nuclear positivity in almost all tumor cells.Memberanous positivity is weak and discontinuous. Hematoxylin counterstain, x100.

c.

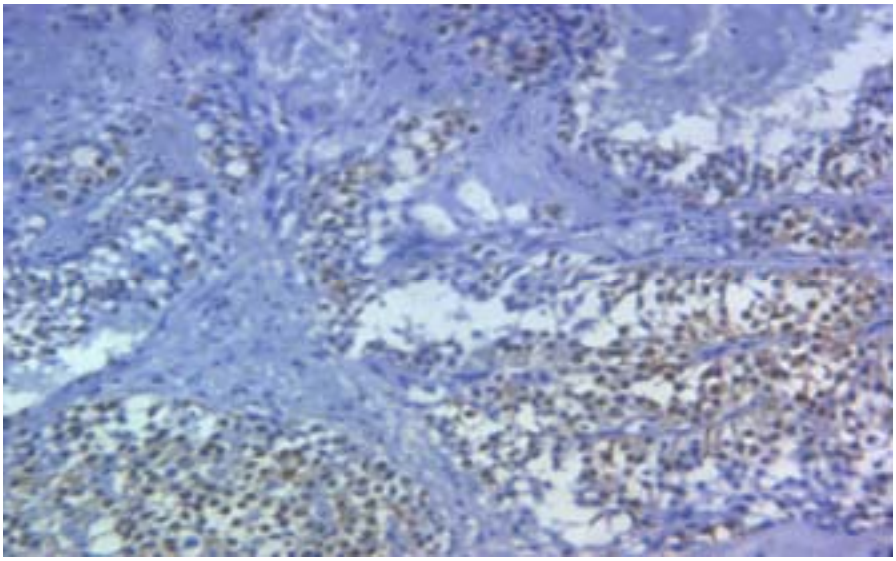
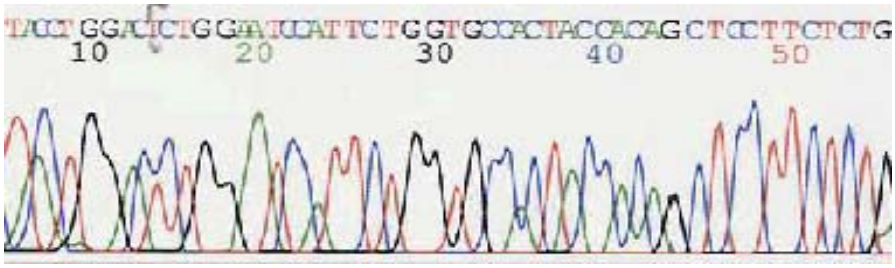


Figure 2 Immunostainning with β-catenin

c. β-catenin immunostain in pseudopapillary areas. Hematoxylin counterstain, x100

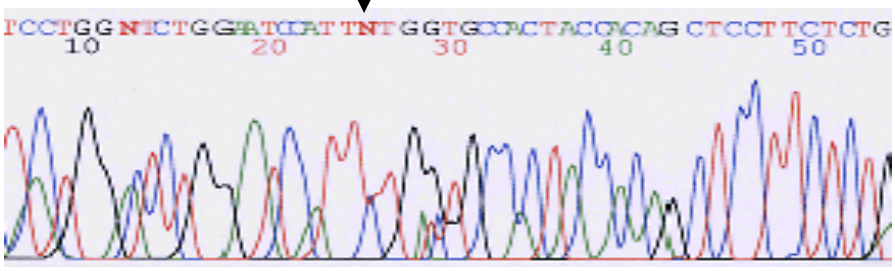
(a)

TCT→CCT



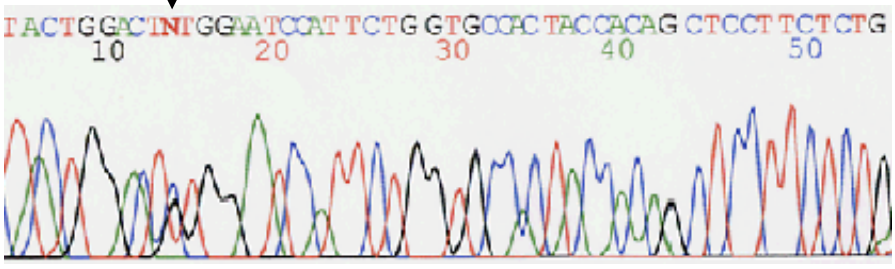
(b)

TCT→TTT



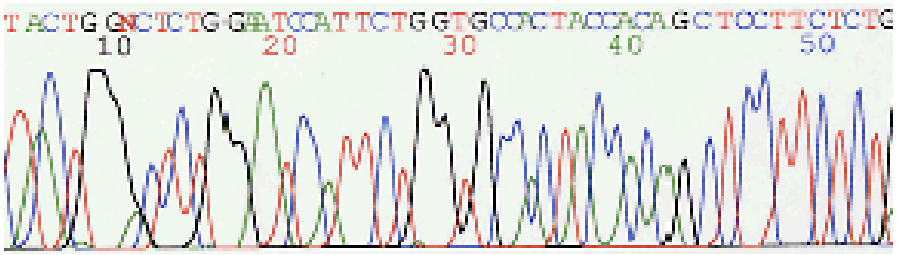
(c)

TCT→TGT



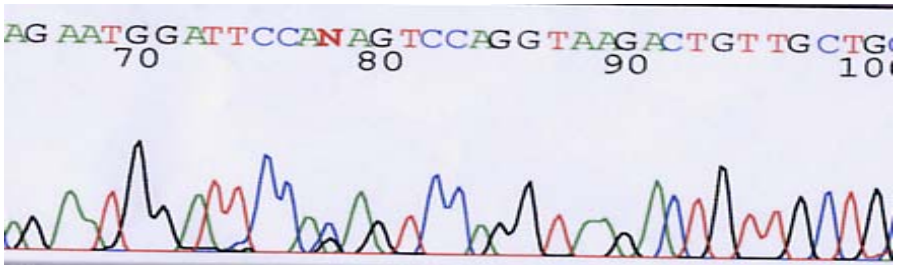
(d)

GAC→GGC



(e)

TCT→TGT



(f)

TCT→CCT

(g)

GAC→GGC

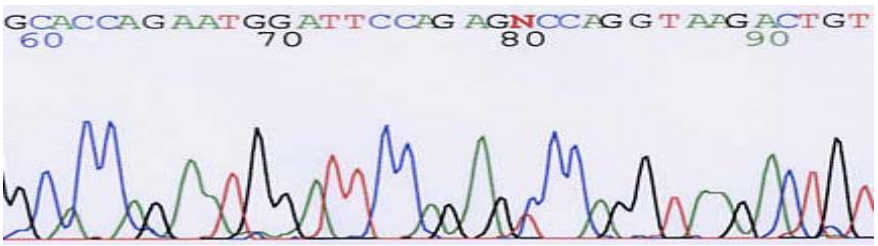


Figure 3. Direct sequencing showing point mutation of Exon-3 of β-catenin gene. Electropherogram indicating mutation at codon 32 to 37 in the tumor sample. Representative DNA sequencing chromatograms demonstrated mutation in Forward sequence: (A) a TCT(Serine) →CCT (Proline) mutation in codon 33 of case 1 (B) a TCT(Serine) →TTT (Phenylalanine) mutation in codon 37 of case 4. (C) a TCT(serine) →TGT (Cysteine) mutation in codon 33 of case 3. (D) a GAC(Aspartic acid) →GGC(Glycine) mutation in codon 32 of case 9 . In reversed sequence (E) a TCT(Serine) →TGT(Cysteine) mutation in codon 37 of case 10. (F) a TCT(Serine) →CCT(Proline) mutation in codon 32 of case 17. (G) a GAC(Aspartic acid) →GGC(Glycine) mutation in codon 32 of case 20.

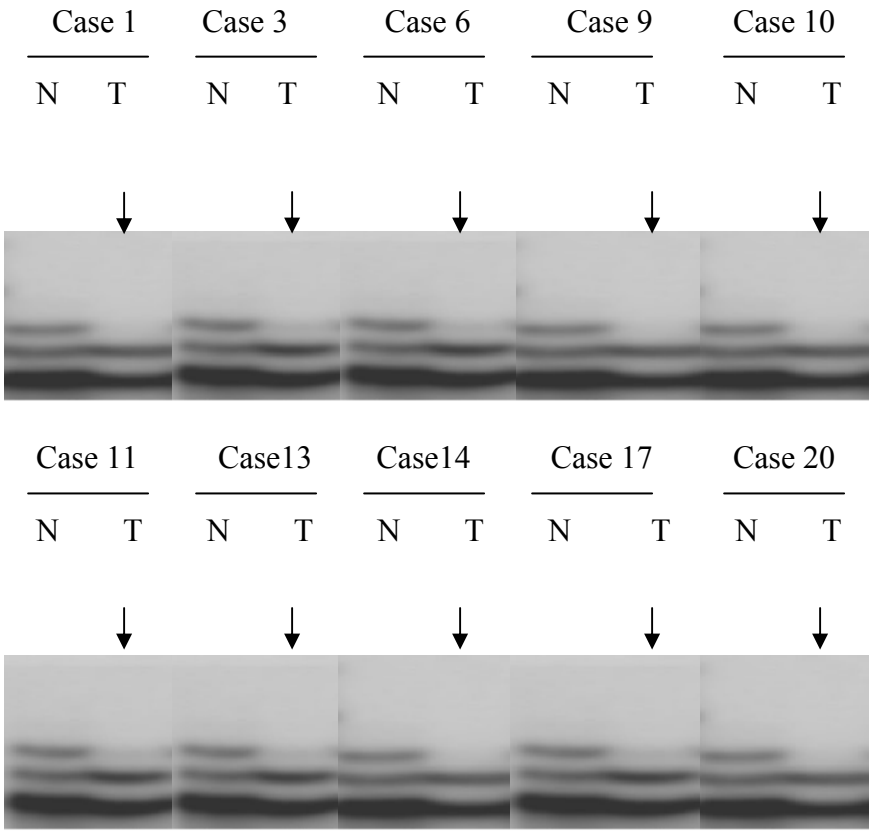


Figure 4. Allelic loss on 5q 22.1 in SPTs. LOH of D5S592 is present in cases 1,3,6,9,10,11,13,14,17,20. N: normal tissues , T: tumor . Arrows, the position of the deleted LOH.

IV. DISCUSSION

Studies of SPTs to date have demonstrated a lack or rarity of genetic alterations commonly present in ductal adenocarcinomas, including mutations in the K-ras oncogene and in p53 and DPC4 tumor suppressor genes.[30-38] The specific molecular alterations that do characterize SPTs remain obscure.[10-14, 19, 28]

Histopathological and immunohistochemical features of SPTs are similar to pancreatoblastoma and acinar cell carcinomas, two other distinctive pancreatic malignancies.[15, 16, 26-28] Although SPTs, pancreatoblastomas, and acinar cell carcinomas occur most frequently in young women, the pediatric population, and in adults, respectively, occasional cases of SPTs have been seen in males and old adults, pancreatoblastomas have infrequently occurred in adults, and acinar cell carcinomas do appear in children. Previous studies of these 3 tumors have demonstrated mutations in the APC/β-catenin pathway, a molecular genotype that is distinct from that of pancreatic ductal adenocarcinoma.[13,14,17-23] The histological and clinicopathological similarities among the 3 tumor types suggest that these neoplasms may share similar genetic alterations.

Similar to two previous studies, the most common molecular alteration in SPTs was a mutation in exon-3 of β-catenin.[13, 14] We showed that 16 of 20 SPTs (80%) harbored β-catenin gene mutations. β-catenin

protein functions both as a submembranous component of the cadherin-mediated cell adhesion system and as a downstream transcriptional activator in the Wnt signaling pathway.[19, 20] Normal β-catenin degradation is promoted by the APC tumor suppressor protein, which, in conjunction with glycogen synthase kinase-3β (GSK-3β) and Axin, promotes phosphorylation of β-catenin on serine/threonine residues that are encoded in exon 3 of the β-catenin gene. Normal levels of β-catenin are maintained by this phosphorylation, which targets β-catenin for subsequent ubiquitin-mediated degradation. Abnormal accumulation of β-catenin and loss of β-catenin regulatory activity can result from stabilized β-catenin mutations or AXIN mutations. For example, most gastric adenocarcinomas can be shown to contain β-catenin mutations.[21, 22] Our results are similar to the work of Susan C and coworkers and somewhat different from that of Tanaka and colleagues. The mutations of β-catenin in exon-3 in our studies varied from codons 32 to 37, while in Tanaka and colleagues' work, it varied from codons 32 to 41.[13,14] All β-catenin mutations detected in the SPTs in this study were 1-bp missense mutations that affected either serine phosphorylation residues in codons 33 and 37 or residues immediately surrounding phosphorylation sites in codons 32 and 34. These mutations therefore would be predicted to interfere with normal phosphorylation and degradation of β-catenin protein.

In addition to the high frequency of β-catenin gene mutations in SPTs,

we observed abnormal nuclear and cytoplasmic β-catenin accumulation in all SPTs, including the 4 SPTs that did not contain identifiable β-catenin mutations. These results are in agreement with earlier studies.[13, 14, 23] Accumulation of cytosolic β-catenin is due to its interaction with T-cell transcription factor (Tcf)/lymphoid enhancer-binding factor (Lef) and translocation of the β-catenin-Tcf/Lef complex to the nucleus.

Recently, researchers studying pancreatic neoplasms have reported that LOH may occur at chromosomal-specific polymorphic sites in DNA extracted from tumor, when compared with DNA from matched normal tissues. In ductal cancer of the pancreas, LOH at 5q, 17p, 9p, 18q, and 13q has been defined.[26, 27] In papilla of Vater cancer, LOH at 5q, 17p, 9p, and 18q has been confirmed.[26, 27] In intraductal tumors, LOH at 5q, 17p, 9p, 18q, and 6q has been observed.[28]

The rarity of SPTs has previously prevented detailed investigation of their molecular pathogenesis. Until now, little or no data about allelic loss on chromosome have been available. In our series, for the first time, we observed a high frequency of 5q 22.1 LOH (10/18, 55.5%) with the microsatellite marker D5S592, while no allelic loss was found at 1p, 5q, 6q, 9p, 9q, 11p, 11q, 17p, or 22q. The high frequency of LOH rates occurring in other pancreatic neuroplasms was not found in our series, such as that for 6q, 9p, and 17p.[29, 30, 31] The LOH rate at D5S592 in this study is similar to that

papilla of Vater cancer (55.5% vs 75%), but is higher than that in ductal cancer (55.5% vs 20%).

In summary, the presence of β-catenin exon-3 mutation and nuclear accumulation in SPTs suggest that this mutation and accumulation arise in tumorigenesis. Frequency of allelic loss on chromosome 5q 22.1 suggests that a locus on DS592 might be involved in the molecular pathogenesis of SPTs. Further study should involve more intensive screening of the microsites on chromosome 5q.

V CONCLUSION

The presence of β-catenin exon-3 mutation and nuclear accumulation in SPTs, suggests that these mutation and accumulation arise in tumorigenesis. In addition, frequency of allelic loss on chromosome 5q suggest that a locus on DS592 might be involved in the molecular pathogenesis of SPTs.

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ABSTRACT IN KOREAN

국 문 요 약

췌장고형가성종양의 분자생물학적연구

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의 과 학 과

고형가성유두종양은 희귀한 췌장신생종양으로써 저악성, 잠재성종양으로 알려져 있다. 그러나 SPT 의 발생 및 그에 참여하는 인자들의 분포는 아직 확실하지 않다. SPT 의 초기 발생 기전을 알아보기 위하여 본 실험에서는 대조군과 SPT 실험군의 췌장조직을 취하여 β-catenin 의 돌연변이, 핵에서의 축적 및 이형접합손실 등을 비교해보았다. 각각 20 개의 SPT 와 대조군의 체장조직에서 β-catenin 의 exon-3 돌연변이 및 아홉번째 염색에서의 LOH 변화를 측정하였다. 동시에 면역조직염색법으로 핵에 축적한 β-catenin 을 발현시켰다. 실험 결과, 16 (80%) 개의

SPT 조직에서 β-catenin 의 exon-3 가 codon 32 부터 codon 37 부분에서 변이를 일으킨 것이 관찰되었다. 한편, β-catenin 은 모든 핵에서 다 축적되는 것으로 관찰 되었다. 또한 10 (18) 개의 조직에서는 5q 대립유전자가 손상되었고, 1p, 6q, 9p, 9q, 11p, 11q, 17p, 22q 부분에서는 그 어떠한 변화도 일으 phosphorylation 키지 않은 것으로 관찰되었다.이와 반대로 대조군에서는 상술한 현상들이 나타나지 않았다.

이로부터 β-catenin 의 exon-3 변이 및 핵에서의 축적은 SPT 종양발생에 관련이 있는 것으로 보여진다. 또한 염색체 5q 의 손상으로부터 D5S592 이 유전자도 SPT 의 발병기전에 참여하는 것으로 추정이 된다.

핵 심 되 는 말: 췌장고형가성유두종양, β-catenin , 돌연변 ,
면역조직염색법, loss of heterozygosity.